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Review

DNA vaccines against influenza

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Genetic vaccine technology has been considerably developed within the last two decades. This cost effective and promising strategy can be applied for therapy of cancers and for curing allergy, chronic and infectious diseases, such as a seasonal and pandemic influenza. Despite numerous advantages, several limitations of this technology reduce its performance and can retard its commercial exploitation in humans and its veterinary applications. Inefficient delivery of the DNA vaccine into cells of immunized individuals results in low intracellular supply of suitable expression cassettes encoding an antigen, in its low expression level and, in turn, in reduced immune responses against the antigen. Improvement of DNA delivery into the host cells might significantly increase effectiveness of the DNA vaccine. A vast array of innovative methods and various experimental strategies have been applied in order to enhance the effectiveness of DNA vaccines. They include various strategies improving DNA delivery as well as expression and immunogenic potential of the proteins encoded by the DNA vaccines. Researchers focusing on DNA vaccines against influenza have applied many of these strategies. Recent examples of the most successful modern approaches are discussed in this review.

Key words: DNA vaccine, influenza, hemagglutinin, immunization, adjuvant, cross-protection

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INTRODUCTION

The concept of DNA vaccines has been experimentally proven about two decades ago (Fynan *et al.*, 1993; Mor, 1998). Since that time they were shown to induce potent T- and B-cell immune responses against a variety of antigens (Khan, 2013). This intriguing strategy of vaccination is based on the following principles: (i) a gene encoding an antigen can be expressed in transfected cells and (ii) a foreign antigenic protein produced within the host cells can induce humoral and cellular immune responses. When a DNA vaccine (consisting of an animal expression plasmid and a carrier) is injected into the tissue, the plasmid molecules have to overcome a great obstacle of cell membrane and next, they must be transported into the nucleus. Both, the somatic cells (e.g. myocytes, keratinocytes) and the nearby antigen presenting cells (APCs) can be transfected at the immunization site. Production of the vaccine protein relies on transcription and translation of the administered vaccine DNA within the transfected cells (Fig. 1). Thus, the foreign antigenic proteins are endogenously produced, intracellularly processed and their fragments (antigenic peptides) are exposed on the cell surface by the MHC class I and class II molecules involved in induction of cellular and humoral immunity. Three mechanism of immunogenicity of DNA vaccines are possible: (i) presentation of antigens by myocytes or keratinocytes to $CD8^+$ cells directly through their MHC class I pathway, (ii) direct transfection of APCs and (iii) phagocytosis of transfected somatic cells by APCs which present the antigen to T cells. For the details of the mechanism of DNA vaccines action the reader is referred to several excellent reviews (Iurescia *et al.*, 2014; Khan, 2013; Kutzler & Weiner, 2008; Li *et al.*, 2012b; Liu, 2011; Moss, 2009).

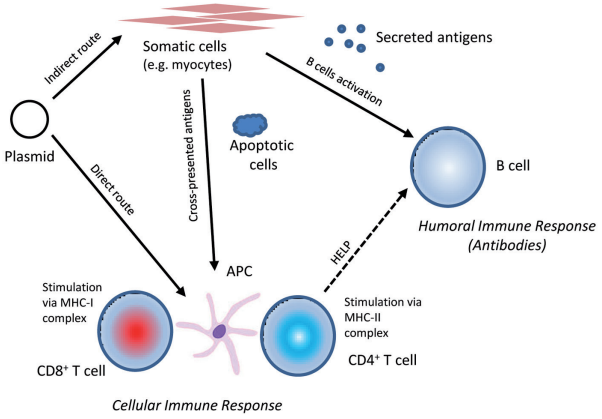

Figure 1. The overall mechanism of DNA vaccine action.
Plasmid DNA can be transfected into antigen presenting cells (APCs) or somatic cells. Thus created protein antigen can be secreted from the cell and activate B cells, leading to antibody production, or activate APCs. APCs are activated by direct transfection or cross presentation of antigens, and migrate to the draining lymph nodes, where antigenic peptides are presented by major histocompatibility complexes (MHC I and MHC II), and stimulates T cells. Modified according to (Iurescia *et al.*, 2014; Xu *et al.*, 2014).

DNA vaccines, as all innovatory preparations, have not only advantages but also disadvantages. Most of pros and cons are listed in Fig. 2, however for the more extensive discussion the reader is referred to the reviews mentioned above. Shortly, this strategy is superior to others mainly due to its simplicity and low cost of production, omitting the steps of propagation and isolation

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Abbreviations: HA, hemagglutinin; M1, matrix protein; M2, ion-channel protein; NP, nucleoprotein; NA, neuraminidase; LPAIV, low pathogenic avian influenza virus; HPAIV, highly pathogenic avian influenza virus; LAIV, live attenuated influenza vaccine; MIV, monovalent inactivated vaccine; HI, hemagglutination inhibition; SPF, specific pathogen-free; PEG, polyethylene glycol; NP, nanoparticles; VLP, virus-like particle; LEC, linear expressing cassette.

of antigens. In case of viral diseases, when the pathogen's proteins are produced by host cells, DNA vaccination leads to immunization with an antigen completely the same as during natural infection. It is also worth to clarify that the often mentioned threats (disadvantages) of DNA vaccines, such as a possibility of antibody production against DNA or insertion of foreign DNA into the host genome (genetic transformation), appeared to be unconfirmed or, if detected, remains at an insignificant level (Klinman *et al.*, 2010). Further objections are related to the fact that the efficiency of such vaccines is not as encouraging in humans and large animals as it is in small animal models.

DNA VACCINES AGAINST INFLUENZA

Influenza is one of the most important illnesses in modern world, causing great public health loses each year, due to lack of medicines and broadly protective, long lasting vaccines. Moreover, due to its zoonotic potential and high changeability, it brings a continuous threat of a new global pandemic. Therefore many researches are focused on preparing new solutions for fighting and controlling influenza. DNA vaccines against influenza virus can be divided into two main categories, (i) vaccines for veterinary purposes, mainly against H5N1 for poultry, but also against other influenza strains for pigs or horses and (ii) vaccines for human seasonal and pandemic influenza, including H5N1 prospective pandemic, tested mainly in mice and ferrets, although some clinical trials are already published (Ledgerwood *et al.*, 2011; Smith *et al.*, 2010; Girard *et al.*, 2013). Despite differences in the regulatory requirements for human and animal vaccines, the main course of technology is the same (van Drunen Littel-van den Hurk *et al.*, 2004; Horimoto & Kawaoka, 2006). Currently, inactivated vaccines, prepared from egg-propagated viruses are commonly used for human and animal immunizations against influenza (Kreijtz *et al.*, 2009; Spackman & Swayne, 2013; Capua & Cattoli, 2013). Nevertheless, it is clear that contemporary influenza vaccines should be more flexible, universal and, in particular, they need to be produced faster, in case of a new influenza outbreak. Such requirements stimulate development of various "new generation" vaccines against influenza virus. Subunit vaccines and DNA vaccines against influenza virus are often based on hemagglutinin (HA), the main viral antigen. Other viral proteins are used rather rarely, usually together with HA, however the sequences encoding NA, M2, NP and M1 were also used in some DNA vaccines (Lim *et al.*, 2013; Patel *et al.*, 2012b). These proteins are usually less immunogenic than HA and are insufficient to obtain the protective response, particularly in case of highly pathogenic avian influenza viruses (HPAIV), however some exceptions were reported (Shen *et al.*, 2012). Frequently, these less immunogenic, yet also less variable, antigens are used to complement the responses and make them less specific to recognize a broader spectrum of viruses, thus more suitable for a universal influenza vaccine (Osterhaus *et al.*, 2011). A broad range of immunity can be also achieved by designing the polyvalent DNA vaccines composed of several sequences of HA antigens originating from distinct strains. For example, the cross-immunity and cross-protection were obtained in mice immunized with a DNA vaccine based on the HA sequences from two distinct strains of H5N1 (Gao *et al.*, 2012), or with a trivalent vaccine containing three HA sequences selected from a panel of 17 sequences representing all H5N1 clades (Zhou *et al.*, 2012). In most of the reported cases vectors with a CMV promoter were used to drive the transcription of a gene encoding an antigen, however in many chicken studies the chicken β-actin promoter has been used as well (Williams, 2013).

In this review we focus on recent and most innovative research trends significantly advancing work on the DNA vaccine against influenza virus. The approaches discussed here are schematically shown in Fig. 3. They include: (i) sequence optimizations in order to improve the level of expression and to direct antigen into appropriate cellular compartments, (ii) complexing of DNA with carriers to improve its uptake and stability, (iii) addition of chemical and biological adjuvants of the immune response and (iv) employment of mechanical devices for efficient and precise application and (v) mixed vaccination (Ferraro *et al.*, 2011; Ulmer, 2002; van den

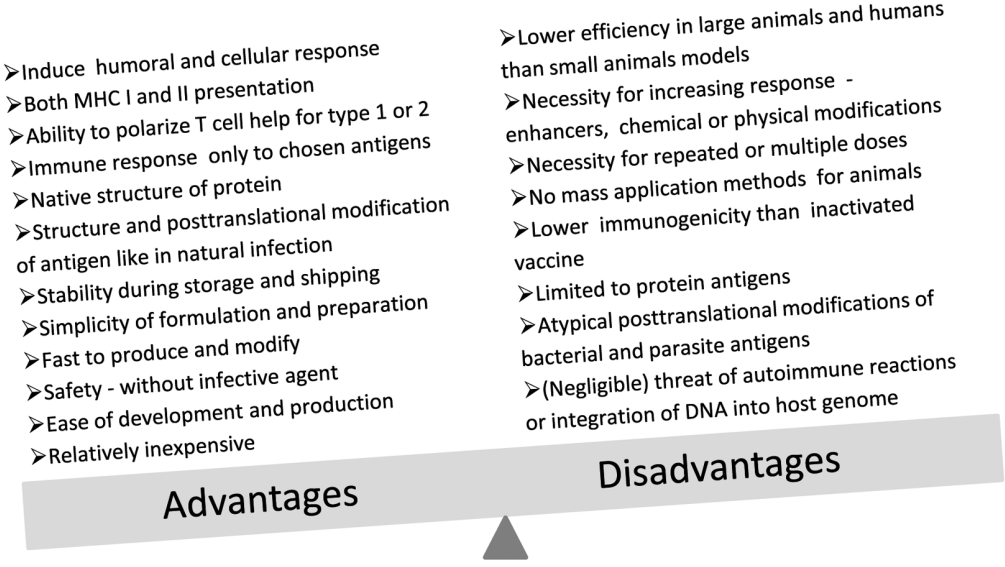

Figure 2. Advantages and disadvantages of DNA vaccines.

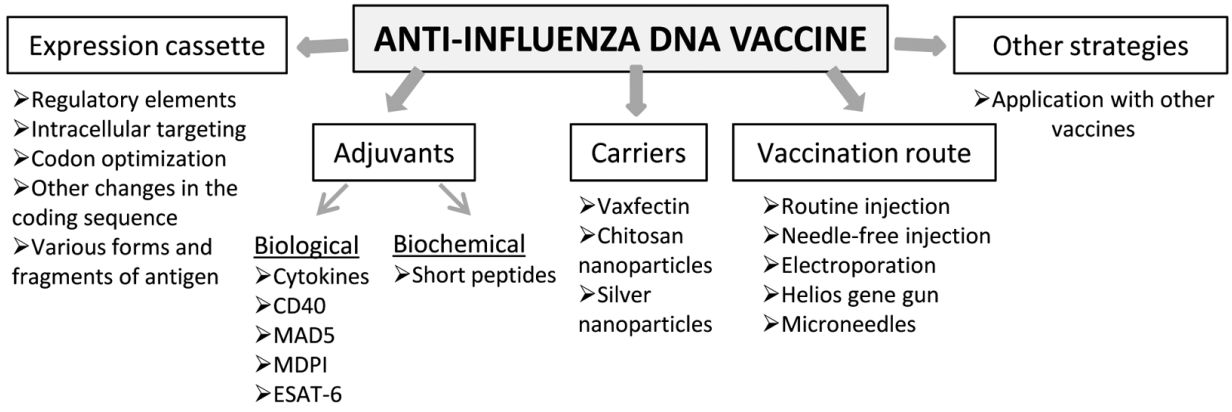

Figure 3. General strategies used to improve efficacy of DNA vaccines against influenza described in this report.

Berg *et al.*, 2010). Some examples of studies described in this report are listed in Table 1 and Table 2.

SEQUENCE MODIFICATIONS

Codon optimization

Codon optimization strategy is used broadly in the development of DNA vaccines against influenza virus. This approach is facilitated by recent advances in bioinformatics methods, resulting in availability of new programs enabling effective sequence adaptation. Codon optimization has been mentioned in many reports, however details of sequence modifications are often omitted and no wild type sequence is used in immunizations for comparison. For example, rabbits immunization with DNA vaccines based on different HA (from H5N1) using sequence optimized to the mammalian system resulted in a potent production of anti-HA antibodies with broad cross reactivity (Wang *et al.*, 2011). Similarly, positive effects (detectable humoral and cellular responses and protection against challenge with a homologous virus) were observed in mice immunized against H5N1 HPAIV and in ferrets immunized against seasonal H1N1 using a codon-optimized HA-based DNA vaccine (Patel *et al.*, 2012a). A successful immunization of Japanese quails with the DNA vaccine based on HA from H5N1 HPAIV and containing sequence optimized for chicken codon usage was reported (Li *et al.*, 2012a). The birds obtained three intramuscular doses of the plasmid (10, 15, 30 or 60 μg) and the challenge experiment with a homologues virus was conducted two weeks later. Antibody responses measured by hemagglutinin inhibition (HI) test showed 100% of seroconversion in the groups immunized with 30 and 60 μg and 60–70% of seroconversion in the groups immunized with 10 and 15 μg of DNA. The mortality (20%) after the challenge was observed only in the lowest dose group. An apparent very good efficacy of this DNA vaccine formulation is clearly emphasized by the fact that all surviving birds were completely protected and no virus shedding or clinical signs of the infection were observed.

In the case when the efficiency of the HA codon-optimized, DNA vaccine against low pathogenic (LP) H6N2 virus was compared to the DNA vaccine based on the wild type HA, no significant differences were observed in responses of chickens to the optimized and not optimized variant of the DNA vaccine, regardless of the dose (Shan *et al.*, 2011). The optimized sequence was shifted to the codon usage bias optimal for chicken (resulting in 74.8% similarity to the wild type nucleotide sequence).

Also in our studies, effectiveness of a DNA vaccine based on H5 HA and containing codons optimized to the chicken host has been demonstrated (Stachyra *et al.*, 2014). Nevertheless, our subsequent chicken immunization studies with the wild type sequence and the sequence with optimized codons failed to prove that the optimized sequence is superior over the wild type sequence in mounting the immune responses (data not published).

Table 1. Selected DNA vaccination studies against influenza performed in a mouse model.

Subtype of the template virus	Strategy for improvement	Reference
H5N1	Codon optimization, rare peptides as an adjuvant	Patel *et al.*, 2012a
H5N1	CD40 gene as an adjuvant	Chen *et al.*, 2013
H3N2	Chitosan nanoparticles as a carrier	Zhao *et al.*, 2011
H3N8	Codon optimization, needle-free application	Ault *et al.*, 2012
H5N1	Electroporation application	Xu *et al.*, 2011
H1N1, H3N2	Head-less HA, electroporation application, VLPs boost	Steel *et al.*, 2010
H1N1, H1N1	Linear cassette, electroporation application	Shen *et al.*, 2012
H5N1	Consensus HA, point mutations, electroporation application	Chen *et al.*, 2011
H5N1	Cleavage site deletion, micro needle application	Kim *et al.*, 2012
H5N1	Point mutations, VLPs boost	Lin *et al.*, 2012

Table 2. Selected DNA vaccination studies against influenza performed in a chicken model.

Subtype of the template virus	Strategy for improvement	Reference
H6N2	Codon optimization, electroporation application	Shan *et al.*, 2011
H5N1	Codon optimization, cleavage site deletion, Lipofectin as a carrier	Stachyra *et al.*, 2014
H5N1	IL-15 and IL18 genes as an adjuvant	Lim *et al.*, 2012
H5N1	IL-15 and IL18 genes as an adjuvant	Lim *et al.*, 2012
H5N1	MAD5 gene as an adjuvant	Liniger *et al.*, 2012
H5N1	MDPI gene as an adjuvant	Jalilian *et al.*, 2010
H5N1	ESAT-6 gene as an adjuvant	Oveissi *et al.*, 2010
H5N1	Silver nanoparticles as a carrier	Jazayeri *et al.*, 2012
H5N1	Electroporation application	Ogunremi *et al.*, 2013

In summary, the advantages of codon optimization approaches are still somewhat controversial.

Other modifications

Other frequently reported sequence modifications (mainly within the HA gene) used in DNA vaccines against influenza include: (i) deletions of the signal peptide or the transmembrane region (Wang *et al.*, 2011), (ii) removing of the proteolytic cleavage site, which is related to the virulence (Kim *et al.*, 2012; Stachyra *et al.*, 2014), (iii) point mutations changing single amino acids to achieve a better cross reactivity (Chen *et al.*, 2011), (iv) point mutations changing glycosylation sites (Lin *et al.*, 2012), (v) using antigen fragments, such as single domains or the stem region (Steel *et al.*, 2010) and (vi) creating a synthetic gene based on the consensus sequence obtained by comparison of sequences available in databases (Chen *et al.*, 2011). Unfortunately, none of these approaches achieved a significant breakthrough concerning the efficacy of a DNA vaccine.

ADJUVANTS

Peptides

Some novel formulations of the adjuvants suitable for DNA vaccines were recently proposed (Patel *et al.*, 2012a). For example, short polypeptides (rare or non-existing in vaccinated organisms) were designed after *in silico* screening of the UniProt database. They were used as adjuvants of the DNA vaccine for mice based on H5 HA. Combination of the DNA vaccine with one of the discovered pentamers (KWCEC) was significantly more effective than the HA plasmid alone. A subsequent challenge experiment indicated complete (100%) protection. Similar results were also observed in ferrets, using the same pentamer and the plasmid with H1 HA sequence. The authors claim that rare amino acid sequences could act as immune modulators positively contributing to the antigen-specific immune activation, however, the mechanism is not further explained.

Cytokines

Various cytokines or their expression cassettes have been applied as biological adjuvants of anti-influenza DNA vaccines (van Drunen Littel-van den Hurk *et al.*, 2004). For example, combinations of the DNA sequence encoding HA from H5N1 HPAIV with the cDNAs encoding either chIL-15 or chIL-18, known to induce the inflammatory response, were administered intramuscularly (in two doses) to chickens (Lim *et al.*, 2012). The group inoculated with a mixture of the plasmids encoding HA and chIL-15 gave the best results in the hemagglutinin inhibition (HI) test. Later, the same team immunized chickens with combinations of plasmids encoding chIL-15 or chIL-18 with plasmids encoding N1 NA or NP (Lim *et al.*, 2013). Both plasmids encoding interleukins improved responses against N1 NA and NP, however, the highest immunological potential had the combination of the plasmids encoding N1 NA and IL-15. Additional *in vitro* results indicated that the plasmids were stable in the muscles of immunized chickens for as long as 6 week after vaccination. Surprisingly, expression of the vaccine antigens was also detected by real time PCR in spleens.

Other biological adjuvants

Proteins involved directly or indirectly in the immunological response, in stress and danger signaling, are good candidates for adjuvants of immunological reactions. DNA fragments encoding four such novel proteins were applied in DNA vaccines against influenza.

The first example is CD40, a member of the tumor necrosis factor receptor family, expressed in many cells and playing an important role in regulation of humoral and cell-mediated immune responses. Mice immunization with the plasmids encoding HA from H5N1 HPAIV in combination with the plasmid encoding CD40 was recently reported (Chen *et al.*, 2013). Vaccine formulations containing the CD40 encoding plasmid induced much higher humoral responses than vaccine formulations with HA encoding plasmid alone. Additionally, an observed increase in expression of genes for Th2 cytokines, namely IL-2 and IL-6, confirms that CD40 indeed positively affected the humoral response.

Efforts of another group have been concentrated on chicken MAD5, a pattern recognition receptor (PRR) which senses danger signals associated with a virus infection and contributes to the activation of signaling and to the secretion of type I IFN (Liniger *et al.*, 2012). The sequence encoding N-terminal part of chMAD5 was cloned into an expression vector as a bicistronic expression plasmid with the sequence encoding HA from H5N1 HPAIV and used to immunize SPF chickens with two suboptimal doses. Vectors expressing either HA or chMAD5 alone were applied as a control. Co-expression of chMAD5 with HA led to considerable amplification of the anti-HA humoral response, high protection level (5 per 6 chickens survived the challenge with a homolo-

gous virus) and reduced virus shedding in tracheal and cloacal swabs.

Mycobacterial DNA-binding protein I (MDPI) acting as an immunodominant antigen stimulating responses via TLR9 pathway was tested in a vaccine against H5N1 for chickens (Jalilian *et al.*, 2010). Triple intramuscular vaccination of chickens with plasmids encoding MDPI and HA (in a combination or each alone) indicated higher humoral responses in the group immunized with combination of the plasmids. Although the observed differences in humoral response (ELISA and HI) were statistically insignificant, the authors proposed that usage of the MDPI-encoding gene as a genetic adjuvant could be beneficial.

In a similar study, combination of the plasmid containing mycobacterial ESAT-6 gene encoding a protein involved in bacterial virulence and the plasmid encoding H5 HA was tested in chickens (Oveissi *et al.*, 2010). All chickens were seropositive in the group that received the plasmid combination, while only 3 out of 8 chickens were positive in the group vaccinated solely with the plasmid encoding HA. Statistically significant differences were also found in the HI titers.

CARRIER FORMULATIONS

One of the strategies reported, was an intramuscular immunization of mice with a DNA vaccine encoding HA from H3N2 swine influenza virus encapsulated in chitosan nanoparticles (Zhao *et al.*, 2011). The naked HA-encoding plasmid was injected as a control. Serum IgG titer (tested by ELISA) was significantly higher in the group vaccinated with nanoparticles than in the group vaccinated with the naked plasmid. Furthermore, the lymphocyte proliferation test indicated that nanoparticles also enhanced cellular immunity. The nanoparticles showed no cytotoxicity during *in vitro* tests. In our studies, DNA vaccine based on HA from H5N1 HPAIV with the commercially available liposomal carrier (Lipofectin, Life Technologies, USA) was successfully used for immunization of chickens (Stachyra *et al.*, 2014). Antibody response measured by ELISA and the HI test indicated an obvious advantage of vaccination with the carrier. Moreover, two doses of such adjuvanted vaccine were sufficient to protect SPF chickens against the challenge with homologous and heterologous (distinct clade) HPAIVs, however, non adjuvanted vaccine was not tested in the challenge experiments. Silver nanoparticles were used as carriers in a DNA vaccine based on H5 HA, applied orally to chickens (Jazayeri *et al.*, 2012). They were composed of Ag covered with PEG and complexed with a DNA plasmid expressing HA. Not only immunological responses but also presence of the nanoparticles in chicken intestinal mucosa was tested for a limited time of 48 hours. Interestingly, the H5 mRNA was detected in tissue samples as early as 1h post immunization. Cellular response was significantly higher (and it was increasing till day 28) in the group vaccinated with HA nanoparticles. Also, the HI titer in this group was high and maintained for a long time. These results indicate huge advantages of nanoparticle approaches in oral applications that might be the most desirable vaccine formulations for mass usage in the poultry industry.

Very good results of clinical trials with anti-influenza DNA vaccines containing Vaxfectin® (Vical, San Diego, CA, USA), a liposomal carrier, were reported (Smith *et al.*, 2010). According to the information provided on the company webpage (http://www.vical.com/technology/vaxfectin/default.aspx) Vical is currently developing several products that utilize Vaxfectin® as an adjuvant, however it is unclear if a DNA vaccine against influenza is one of these potential products.

MECHANICAL DEVICES

Jet injection

Effectiveness of conventional intramuscular injection and a needle-free injection system (PharmaJet, Inc., Golden, CO, USA) was compared in pigs immunized with DNA plasmids carrying the HA sequences from H1N1 (seasonal and pandemic) and H3N2, using monovalent or trivalent formulations (Gorres *et al.*, 2011). The HI titers and virus neutralization were robust and similar in all experimental groups regardless of the route of administration. Groups vaccinated with monovalent vaccines and challenged with pandemic H1N1 strain showed full reduction of virus shedding and lack of histopathological changes in lungs. The authors claimed that the needle-free delivery worked with the same efficacy as the conventional injection. It can be thus an interesting alternative for veterinary and human DNA vaccine applications. Recently, immunization of ponies with monovalent or trivalent DNA vaccines encoding HAs from different H3N8 equine influenza viruses was reported (Ault *et al.*, 2012)2012. Preliminary studies were first conducted in a mouse model and the vaccine efficacy was confirmed by the HI test. Next, ponies received three doses of the vaccine by intramuscular or subdermal applications using the needle-free system (PharmaJet, Inc., Golden, CO, USA). Challenge with a heterologous virus was performed 7 weeks after the third dose and was followed by serological assays, clinical monitoring, virus shedding test and cytokine expression profiling. A certain level of cross-response was obtained in the group immunized by the needle-free device that received the trivalent formulation by subdermal application. Interestingly, antibody titers were slightly higher in the monovalent vaccine group immunized with the needle-free system than with the syringe, which suggested that this way of vaccine administration may enhance immune responses. However, no significant differences between the conventional and needle-free application was observed in case of the degree of protection, virus shedding and clinical symptoms. All of them were significantly reduced in all immunized groups compared to the non-immunized controls. Thus, the needle-free system is at least comparable to the conventional needle vaccination, yet it could be a more convenient alternative for massive vaccinations of larger species.

Jet injection was also one of the routs tested in clinical trials of anti-influenza DNA vaccines (Smith *et al.*, 2010). Plasmids encoding consensus NP and M2 from seasonal strains (H3N2 and H1N1) and HA from H5N1 HPAIV were complexed with Vaxfectin in mono- or trivalent formulations and injected intramuscularly either by the conventional syringe with a needle or by the needle-free device (Biojector 2000, Bioject Medical Technologies, Inc., Portland, OR, USA), at doses ranging from 0.1 to 1 mg. Immunization was repeated 3 weeks later and after 2 additional weeks the humoral serum response was measured as well as the potential adverse effects were monitored. All doses, regardless of the immunization method, were well tolerated without serious adverse effects or discontinuations. The monovalent formulation, containing only the HA gene, was effective (HI titers

>40) at higher doses (0.5 and 1 mg) in both, needle and needle-free applications. The trivalent formulations containing three influenza genes were less effective, probably due to proportionally smaller dose of HA sequences in their composition.

Electroporation

Electroporation is broadly used under laboratory conditions for improving DNA uptake by the cells. It can also improve the immune response by generation of the stress signals in tissues of the vaccinated individual. The weak point of this method is that the electroporation parameters have to be carefully optimized in order to minimize tissue damage while maintaining the high efficacy of DNA uptake (van Drunen Littel-van den Hurk *et al.*, 2004).

Electroporation was successfully used in an experiment with mice immunized with DNA vaccines based on HA and NP from H5N1 where even the cross-reactive response against heterologous H9N2 was achieved (Xu *et al.*, 2011).

Other experiments have been performed using a DNA plasmid with partial HA ("stem" region) from human H1N1 and H3N2 (Steel *et al.*, 2010). Mice were immunized with the DNA vaccine (injection was followed by electroporation), boosted with virus like particles (VLPs) and challenged. Sera from groups immunized with plasmid encoding H1 stem cross-reacted in ELISA with the broad panel of hemagglutinins (H1, H2, H3 and H5). Furthermore, despite the lack of neutralizing antibodies, immunized mice were protected from infection during challenge and had only 6% body weight loss.

Slightly different strategy was used by another group working with a synthetic linear expressing cassette (LEC) encoding the M1 and NP genes from the seasonal H1N1 virus (Shen *et al.*, 2012). Mice received three intradermal doses of the vaccine followed by electroporation. Plasmid vectors containing all genes were also administrated as a control. ELISA test and flow cytometry analysis indicated that electroporation significantly enhanced the response to immunization with LECs. The challenge experiment using homologous (H1N1) and heterologous (H5N1) viruses was performed 10 weeks later, however vaccine effects were not clearly indicated in this study. Nevertheless, a DNA vaccine based on other viral genes than HA can ensure some protection.

Another electroporation study focused on two consensus HA sequences of H5N1 viruses from clade 1 and 2 (Chen *et al.*, 2011). Mice immunization was followed by a challenge. Protection against viruses even from different clades was high (90–100%). These results indicate suitability of the "consensus" approach in development of a universal vaccine against influenza.

Immunization of chickens with a DNA vaccine encoding HA from H6N2 LPAIV was more effective when electroporation was following the intramuscular injections, where the corresponding group immunized by injection without electroporation had an almost 3-fold lower average HI titer (Shan *et al.*, 2011). Interesting results were reported by other researchers, who showed protective immune response in chickens after a single dose immunization with electroporation (Ogunremi *et al.*, 2013). Animals were immunized with a plasmid containing a sequence encoding HA from H5N1 using 10, 100 or 250 μg of the plasmid. An intramuscular injection was immediately followed by electric pulses from a TriGrid electrode (Ichor Medical Systems, San Diego, CA, USA). Results of the HI test and competitive ELISA showed 100% of seroconversion in groups immunized by electroporation with 100 and 250 μg of DNA. On the contrary, groups immunized with the same vaccine doses but without electroporation had low HI titers and none of them was seropositive. The challenge experiments with heterologous H5N2 HPAIV, performed one week after immunization indicated that all chickens immunized with higher doses (100 or 250 μg) with electroporation survived infection. Chickens from these groups showed no clinical symptoms, whereas birds receiving vaccine without electroporation showed a similar pattern of susceptibility as those injected with the control plasmid. It is also worth to mention that all seropositive birds, even those that were immunized with 10 μg dose of the vaccine, turned out to be protected against the challenge. This study showed a really promising perspective for reducing the number of the doses of the protective DNA vaccine by application of electroporation.

Other devices

Immunization of rabbits with plasmids containing two variants of an optimized HA gene, based on HP H5N1, via Helios gene gun (BIO-Rad, USA) has been reported (Wang *et al.*, 2011). Animals received three doses of plasmid DNA into a shaved abdominal skin. Humoral responses were detected by serological assays (ELISA, HI and micro neutralization). Considering a relatively small dose of plasmid DNA (36 μg) used per rabbit (average weight of a rabbit is about 2 kg) these results strongly validate effectiveness of immunization with the gene gun. However, this method has many limitations. For example, only relatively small amounts of DNA can be coated by gold beads.

Usage of microneedles for intradermal vaccination is a novel and recent approach (Kim *et al.*, 2012). The DNA vaccine used in this work was based on HA from H5N1 HPAIV. Plasmid DNA (about 3 μg per dose) was used to coat 700 μm-long microneedles (5 needles per array). Mice were immunized (3 times, 5 weeks apart) into depilated skin on the back and an intramuscular injection was performed as a control. The antibody titers (ELISA, HI) were already significantly higher after the second boosting in groups immunized with microneedles. Additionally, a clear difference in the isotyping profile with IgG2a dominant in microneedle application was observed. A challenge with a homologous virus was performed 21 weeks post immunization. A quite high protection level in the group vaccinated with micronedles (67% of survival) was observed, while the group injected conventionally had no resistance — all mice from this group died on the 8th day post challenge.

OTHER STRATEGIES

Combination of a DNA vaccine with other vaccine formulations is often beneficial. DNA vaccines are mostly used as a "primer" followed by a boost with other kinds of vaccines. A good example of such approach is the recently reported immunization of mice with a DNA vaccine encoding H5 HA, followed by application of VLPs consisting of HA, NA, M1 and M2 produced in a baculovirus system (Lin *et al.*, 2012). Analysis of the HI titers indicated significantly better results for DNA–VLP schedule in comparison to DNA–DNA or VLP–VLP vaccinations.

VLPs were also used as a boost immunization in the previously mentioned report (Steel *et al.*, 2010). Briefly, the results were good, but no control DNA immunizations was done in this experiment. Another group boosted their DNA vaccine with live attenuated and inactivated virus particles (LAIV) in ferrets studies (Suguitan *et al.*, 2011). Primarily, a set of recombinant H5N1 influenza viruses from different clades were prepared as LAIV formulations for the "booster" dose, while an optimized (no details provided) HA sequence was cloned into the respective expression vector. Animals were immunized in various combinations of the prime-boost formulations and then they were subsequently challenged. Humoral responses (ELISA) were very poor in the case of DNA–DNA immunization, while the DNA–LAIV schedule induced even a slightly higher response than LAIV–LAIV or LAIV–DNA immunization. Microneutralization assay with homologous and heterologous viruses confirmed a high potential of this mixed prime-boost regiment. Moreover, after challenge the effective reduction of viral titers in lungs was observed only in the DNA–LAIV and LAIV–LAIV groups. Therefore, DNA priming–LAIV boosting could be a good alternative for two doses of attenuated vaccines, which are very efficient but rather inconvenient due to necessity of live pathogen handling. In another report, mixed immunizations of mice and ferrets using plasmid DNA and adenoviral vector system was described (Rao *et al.*, 2010). Initially, immunogenicity of the conventional plasmid vectors encoding HA, M2 and NP from HP H5N1 was checked in mice and the best combination was chosen. Next, sequences of the target genes were cloned into three separate, replication defective, recombinant adenovirus vectors and the viruses were produced by serial passages in complementing cells. Then, male ferrets were immunized with mixed DNA plasmid vectors at a total dose of 250 μg intramuscularly (3-fold; 3 weeks apart). Three weeks after the last dose of DNA vaccine, the animals were injected with 10^{10} particles of recombinant adenoviruses, and 10 weeks later they were challenged with a homologous virus. A good level of protection and significantly high antibody levels were observed in the groups immunized with plasmids encoding HA alone or with addition of plasmids encoding M2 and NP. Those additional antigens were not able to induce resistance in the absence of HA.

Prime-boost strategy with plasmid DNA and monovalent inactivated vaccine (MIV) was used also in phase 1 clinical trials against HP H5N1 (Ledgerwood *et al.*, 2011). Several schedules were compared, including intramuscular single or double dose with 4 mg of HA DNA followed by immunization with 90 μg of MIV in 4 or 24 week intervals. MIV-MIV immunization was conducted as a control. ELISA, HI and micro neutralization tests indicated a very good level of responses in the case when the time between DNA and MIV doses was long (24 weeks apart). It is also worth mentioning that two doses of DNA with a shorter (4 week) interval resulted in a better response than MIV-MIV vaccination with the same (4 week) schedule.

FUTURE PERSPECTIVES

A continuous progress in the development and application of DNA vaccines is observed. Three DNA vaccines have already been licensed for veterinary purposes (for horses — against West Nile virus, for fishes — against infectious hematopoietic necrosis virus, and for dogs — against canine malignant melanoma). No DNA vaccine for humans is licensed, but many candidates are under clinical evaluations (see: http://clinicaltrials.gov/). Regulatory requirements for DNA vaccines are published in several guidance documents containing recommendations for production and safety evaluation of DNA vaccines intended for use in humans (EMEA, 2001; FDA, 2007; WHO, 2007). There are solid basis for expectations that a DNA or a partial-DNA vaccine product will be approved for human use against pandemic and highly pathogenic H5N1 virus (in case of a future pandemic) in a few years (Ferraro *et al.*, 2011). Additionally, the DNA vaccine for veterinary purposes against avian influenza, and possibly against equine or swine influenza, will be developed. They might be quite important especially for Asian countries, where infections of poultry (and humans) frequently occur. In European countries, due to the current law restrictions, DNA vaccine will be probably initially approved for some special cases, like immunizations of valuable flocks or zoo collections, but not for mass applications (Spackman & Swayne, 2013). Hopefully, work on improvement of the efficacy of DNA vaccines will not stay behind research on other new generation vaccines, and more and more effective and safe strategies will be invented.

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